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Identification by Genomic and Genetic Analysis of Two New Genes Playing a Key Role in Intermediate Glycopeptide Resistance in *Staphylococcus aureus*▽

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Endogenous, low-level glycopeptide resistance in *Staphylococcus aureus* results from multifactorial genetic changes. Comparative genomic hybridization analysis revealed the specific deletion of a 1.8-kb segment encompassing two adjacent open reading frames (ORFs) of unknown function in a teicoplanin-susceptible revertant (strain 14-4rev) compared to the sequence of its isogenic, teicoplanin-resistant parental strain, strain 14-4. This provocative finding prompted us to perform a detailed genetic analysis of the contribution of this genomic segment to glycopeptide resistance. Despite repeated efforts in our laboratory, 14-4 and 14-4rev have proven refractory to most genetic manipulations. To circumvent this difficulty, we evaluated the contribution of both putative ORFs (designated teicoplanin resistance factors *trfA* and *trfB*) on teicoplanin resistance in a different, genetically tractable background. Genetic analysis showed that single or double *trfA* and/or *trfB* mutations abolished teicoplanin resistance in two independent teicoplanin-resistant derivatives of NCTC8325 strain ISP794 generated by two-step passages with the drug. The frequency of teicoplanin-resistant mutants was markedly decreased by the absence of *trfAB* in the teicoplanin-susceptible ISP794 background. Nevertheless, a low rate of teicoplanin-resistant mutants was selected from ISP794 *trfAB*, thus indicating an additional contribution of *trfAB*-independent pathways in the emergence of low-level glycopeptide resistance. Further experiments performed with clinical glycopeptide-intermediate *S. aureus* isolate NRS3 indicated that the *trfAB* mutation could affect not only teicoplanin resistance but also vancomycin and oxacillin resistance. In conclusion, our study demonstrates the key role of two novel loci in endogenous, low-level glycopeptide resistance in *S. aureus* whose precise molecular functions warrant further investigation.

Glycopeptide antibiotics are first-line agents for the treatment of methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA) infections, but there is growing concern about the emergence of glycopeptide-resistant isolates. Glycopeptides (vancomycin and teicoplanin) inhibit cell wall synthesis by binding to the C-terminal D-alanyl–D-alanine (D-Ala–D-Ala) residues of cell wall precursors and nascent peptidoglycan, which blocks the actions of glycosyltransferases and transpeptidases (16, 36). Seven clinical isolates showing high-level glycopeptide resistance (MICs ≥ 16 μg/ml) have been identified in the United States (37, 50). The highly vancomycin-resistant phenotype is based on the acquisition of the exogenous *vanA* complex from *Enterococcus faecalis*. The *vanA*-associated mechanism of resistance in *E. faecalis* involves the production of cell wall precursor molecules with altered C-terminal D-Ala–D-Lac residues which are not recognized by vancomycin. Through the acquisition of the *vanA* gene complex, *S. aureus* is also capable of producing C-terminal D-Ala–D-Lac residues that may inhibit glycopeptide binding (44, 49).

Since 1997, clinical isolates with low-level glycopeptide resistance (vancomycin MICs, ≥4 to <16 μg/ml) have been reported and are referred to as glycopeptide-intermediate *S. aureus* (GISA) isolates (11, 45, 46). The mechanism of resistance observed in GISA isolates is considered endogenous and results from multifactorial mutations that are gradually selected by exposure to glycopeptides. Frequently reported phenotypic alterations of some, but not all, GISA strains are cell wall thickening and alterations in colony size and pigmentation. Biochemical studies of several GISA strains indicated an increased proportion of nonamidated muropeptides and D-Ala–D-Ala-free residues, which were linked with a reduction of cell wall cross-linking and alterations in cell wall turnover (10, 17, 45).

In recent years, several molecular studies have identified the genes involved in GISA resistance (9, 24, 28, 30, 32, 40, 42). Some of the genes revealed by transcriptomic analyses, such as *vraRS*, *graRS*, *tcaA*, and *ccpA*, were subsequently confirmed by genetic analysis to contribute to glycopeptide resistance (9, 19, 24, 29, 31, 35, 43). However, the functional links between these different pathways are still elusive, and no global model of the molecular mechanisms of glycopeptide resistance has been provided.

Previous studies in our laboratory showed the altered expression and regulation of some major virulence genes in a teicoplanin-resistant derivative of a clinical MRSA isolate compared to the expression and regulation of the genes in its teicoplanin-susceptible parent and a teicoplanin-susceptible

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TABLE 1. Strains used in this study

Strain or plasmid	Relevant genotype or characteristic	Source or reference
S. aureus		
MRGR3	Tei^s parent	Lucet et al. (26a)
14-4	Tei^r derivative of MRGR3	Vaudaux et al. (48)
14-4rev	Tei^s revertant of 14-4	Renzoni et al. (41)
RN4220	8325-4; r$^-$ m$^+$	R. Novick
ISP794	8325 *pig-131*	Stahl and Pattee (45a)
AR376	ISP4-2-1	This study
AR612	AR376 Δ*trfA*::*tetK*	This study
AR618	AR376 Δ*trfB*::*ermB*	This study
AR532	AR376 Δ*trfAB*::*tetK*	This study
AR548	AR376 with insertion of a kanamycin resistance determinant in the intergenic region between SA0859 and SA0860	This study
AR627	AR612 with *trfA* restored, kanamycin resistant, *tetK* negative; Φ80α transductant of AR548	This study
AR628	AR618 with *trfB* restored, kanamycin resistant, *ermB* negative; Φ80α transductant of AR548	This study
AR561	AR532 with *trfA* and *trfB* restored, kanamycin resistant, *tetK* negative; Φ80α transductant of AR548	This study
NRS3	GISA and MRSA, NARSA reference strain	NARSA collection[a]
AR619	NRS3 Δ*trfAB*::*tetK*; Φ80α transductant of AR532	This study
AR596	ISP794 Δ*trfAB*::*tetK*	This study
ALC2057	RN6390 *sarA*::*kan*	Cheung et al. (7)
Plasmids		
pCL84	*tetK* for tetracycline resistance	Lee et al. (26)
pUC19-MuSupF	Bacteriophage Mu transposon vector	Haapa et la. (15)
pEC2	*ermB* for erythromycin resistance	Brückner (4)
pBT2	Thermosensitive shuttle vector, *amp* and *cam* for ampicillin and chloramphenicol resistance	Brückner (4)

[a] http://www.narsa.net/.

revertant, which emerged spontaneously in an experimental model of foreign body infection (41). Subsequently, comparative genome hybridization analysis of this set of closely related strains revealed the specific loss of a 1.8-kb segment which encompassed two adjacent putative open reading frames (ORFs) in the teicoplanin-susceptible revertant compared to the sequence of its teicoplanin-resistant parent. This provocative finding prompted us to perform a detailed genetic analysis of the contribution of this genomic segment to glycopeptide resistance.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. MICs were determined by broth macrodilution according to CLSI methodology guidelines (8). Commercially available antibiotics were used: teicoplanin (Sanofi Aventis), vancomycin (Sandoz), oxacillin (Sigma), and gentamicin (Essex). Strain MRGR3 is a previously described vancomycin- and teicoplanin-susceptible (Tei^s) clinical MRSA isolate for which the average vancomycin and teicoplanin MICs in cation-adjusted Mueller-Hinton broth (MHB; catalog no. 212322; Difco, Detroit, MI) were 1 and 1 to 2 μg/ml, respectively (41, 48). Strain 14-4 is a stable teicoplanin-resistant (Tei^r) derivative of MRGR3 (41, 48) for which the average vancomycin and teicoplanin MICs were 4 and 16 to 32 μg/ml, respectively. Strain 14-4rev is a teicoplanin-susceptible (Tei^s) revertant of strain 14-4 for which the average vancomycin and teicoplanin MICs were 1 μg/ml each (41). Reference strain NRS3 is a clinical GISA provided by the Network of Antimicrobial Resistance in *S. aureus* (NARSA; www.narsa.net). The teicoplanin and vancomycin MICs for strain NRS3 were 8 μg/ml each.

Population analysis. Aliquots (1 ml) of overnight cultures grown in MHB at 37°C were diluted 1:50 in fresh MHB and grown for 3 h at 37°C without shaking. The bacterial cultures were adjusted to a 0.5 McFarland standard (1.5×10^8 bacteria/ml), which corresponded to an optical density at 600 nm of 0.1. Two slightly different methods were used to analyze the populations. (i) Standard population analysis profiles (PAPs) were obtained by spreading 1×10^8 bacteria on Mueller-Hinton agar (MHA) containing different concentrations (2 to 8 μg/ml) of teicoplanin. The colonies were counted after 48 h of incubation at 37°C, and the viable counts were plotted against the teicoplanin concentration. (ii) In the modified PAP method (the spot PAP method), serial dilutions (10^{-1} to 10^{-7}) of the exponential culture adjusted to a 0.5 McFarland standard were prepared, and then aliquots of each dilution (10 μl) were spotted on MHA containing different concentrations of teicoplanin. The relative efficiency of colony formation (ECF) was calculated by normalizing the number of colonies, scored on plates containing antibiotic each at concentration at 48 h, to the number of bacteria obtained on agar without antibiotic.

Selection of teicoplanin-resistant derivatives of ISP794. An overnight culture of NCTC8325 strain ISP794 (MIC = 1 μg/ml) was diluted 1:50 in MHB and was grown for 3 h at 37°C without agitation. The bacterial cultures were adjusted to a 2.0 McFarland standard in 0.9% NaCl, and ca. 3×10^8 CFU was plated on brain heart infusion agar (BHIA) containing increasing concentrations of teicoplanin (0.5 to 16 μg/ml). Ten independent colonies (first-step mutants) were recovered from plates containing 2 μg/ml of teicoplanin, which represented the highest glycopeptide concentration that allowed bacterial growth at 48 h at 37°C under these experimental conditions. To assess the stability of the increase in the teicoplanin MIC displayed by the first-step mutants, each isolate was grown overnight in glycopeptide-free MHB and then retested on teicoplanin-supplemented BHIA, as described above. Several independent second-step mutants that had grown for 48 h on BHIA supplemented with 4 μg/ml of teicoplanin at 37°C were isolated and stored in glycerol at −70°C. Two of these second-step mutants, strains ISP4-2-1 and ISP4-2-4 (teicoplanin MIC = 8 μg/ml) were used for further studies.

Total gDNA extraction and labeling. Genomic DNA (gDNA) was prepared from an overnight culture grown in MHB at 37°C, as described previously (5). Briefly, the bacterial pellets were lysed with 200 μg/ml of lysostaphin (Ambicin; Applied Microbiology, Tarrytown, NY) for 15 min at 37°C, and the gDNA was then isolated with a DNeasy tissue kit (Qiagen). The DNA was quantified by using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE). gDNA (2 μg) from strain MRGR3, 14-4, or 14-4rev was labeled by using the BioPrime labeling system kit (Invitrogen). Briefly, after heat denaturation of the DNA, random primers, a deoxynucleoside triphosphate mix (1.25 mM each dATP, dGTP, and dTTP and 0.6 mM dCTP), Cy3- or Cy5-dCTP (NEN Perkin-Elmer), and Klenow DNA polymerase (40 U) were added. The labeling reaction was carried out at 37°C for 2 h. The labeled gDNA was purified with Centrisep columns (Princeton).

Comparative genome hybridization (CGH) analysis. For microarray hybridization by use of a dual-label experimental approach, equivalent amounts of Cy3-labeled and Cy5-labeled gDNAs were diluted in 250 μl Agilent hybridization

TABLE 2. Primers used in this study

Gene or mutation and primer	Primer sequence (5′–3′)[a]
trfA upF	TCCCCCGGGCAAGTTGGTTATAATGAGGACG
trfA upR	CGGGATCCGCATCATCATCAGACATATTC
trfA downF	ACGCGTCGACGATAGTCATGTTGATCAAGAAG
trfA downR	AACTGCAGTTATTATGACAATAATCAACATC
trfB upF	TCCCCCGGGGGACAAATCGCAAACGTGGCG
trfB upR	CGGGATCCCTTGCTTGCCACACACAGGAC
trfB downF	CGGGATCCCAGATAGAGGTAATGACGTGC
trfB downR	AACTGCAGTTCACCTGCTAGCGTAGCGCC
kan upF	GGGGTACCCATTTATTCTACTTAACTGGTC
kan upR	CGGGATCCCGCTAAACAAAGGGGATGGG
kan downF	CGGGATCCCAGACACGACGTTTTTTTATG
kan downR	AAAACTGCAGGAAATGCCAAGCTCAAAGTAC
tet marker upF	CGGGATCCGCTTCACAGAAATTCTAGAAC
tet marker downR	ACGCGTCGACTTTTATTACCTACAACTTCTTTA
kan marker upF	CGGGATCCGATAAACCCAGCGAACCATTTG
kan marker downR	CGGGATCCATCGATACAAATTCCTCGTAGG

[a] Underlined regions represent restriction enzymes sequences.

buffer and hybridized to a customized StaphChip oligonucleotide array for 17 h at 60°C. The slides were washed, dried under a flow of nitrogen, and scanned (Agilent) as described previously (5) by using a 100% photomultiplier power tube for both wavelengths. All positive and significant local background signals were subtracted by using Feature Extraction software (version A6.1.1; Agilent) and were corrected for unequal levels of dye incorporation or unequal loads of labeled product. The algorithm consisted of a rank consistency filter and curve fitting by use of the default LOWESS (locally weighted linear regression) method. Irregular or saturated spots were excluded from the subsequent analyses. Spots showing a reference signal less than the background signal plus 2 standard deviations were also excluded from the subsequent analyses. Gene homology searches were performed by using the BLAST network at the Swiss Institute of Bioinformatics (http://www.expasy.org/sprot/).

Targeted gene disruption mutagenesis. Chromosomal gene disruption mutants were constructed by deletion of targeted genes and insertion of either tetracycline or erythromycin resistance genes by using the temperature-sensitive vector pBT2 (4). Briefly, approximately 800 bp of the upstream and downstream flanking sequences were amplified with the primer pairs described in Table 2. Targeted disruption vectors were constructed as follows. For the Δ*trfA* mutant, a tetracycline resistance marker was obtained from plasmid pCL84 (26) by a PCR amplification that incorporated BamHI and SalI restriction sites with the primers described in Table 2. For the Δ*trfAB* mutant, the tetracycline resistance marker BglII was obtained by using the pCL84 HindIII fragment ligated with HindIII-BamHI adaptors into the BamHI-digested pUC19-SupFMu vector (15), followed by BglII digestion. For the Δ*trfB* mutant, the erythromycin resistance marker was obtained by BglII digestion of an engineered pEC2 vector variant (4). Plasmids were first electroporated into restriction-defective strain RN4220 and were then transferred by electroporation into ISP4-2-1 by selecting for either tetracycline or erythromycin resistance at 30°C. ISP4-2-1 harboring the recombinant plasmid was grown overnight at 30°C, followed by growth with applied marker selection for 6 days with dilution passages at 42°C, a nonpermissive temperature for pBT2 replication. The bacteria were plated on agar containing 3 μg/ml tetracycline or 5 μg/ml erythromycin and were then replica streaked on plates containing chloramphenicol at 15 μg/ml to screen for chloramphenicol-sensitive colonies. Double-crossover events corresponding to the desired gene disruptions were confirmed by PCR and sequencing. All disruption mutants were backcrossed into ISP4-2-1 by using bacteriophage Φ80α and were reconfirmed by PCR. Strains AR612, AR618, and AR532 were designated Δ*trfA*, Δ*trfB*, and Δ*trfAB* mutants, respectively. The Δ*trfAB* disruption mutants of strains NRS3 (strain AR619) and ISP794 (strain AR596) were obtained by transduction from AR532 by using bacteriophage Φ80α.

***TrfAB* gene restoration.** To restore either or both of the *trfA* or *trfB* mutations with wild-type *trfA* or *trfB* genes, we first premarked strain ISP4-2-1 by targeted insertion of a kanamycin resistance gene approximately 2 kb from the *trfAB* locus in the intergenic region between genes SA0859 and SA0860 using pBT2, as described above. Briefly, 800 bp downstream and 800 bp upstream from chromosomal locations 975455 and 975463 (sequence coordinates by use of the N315 annotation [25]), respectively, were amplified by using the primer pairs described in Table 2. The kanamycin resistance marker was obtained from strain ALC2057 by PCR amplification (7) by the incorporation of BamHI restriction sites (Table 2). Double-crossover events were identified by screening for kanamycin-resistant but chloramphenicol-sensitive colonies. One colony, designated ISP4-2-1Kan (AR548), was selected, and control experiments showed that the presence of the kanamycin resistance gene did not detectably alter the teicoplanin or vancomycin resistance (MIC = 8 μg/ml) compared to that of its parent Tei^r strain, ISP4-2-1. Genetic restoration of the mutant strains was next performed by transduction with bacteriophage Φ80α lysates of AR548 by selecting for the tightly linked kanamycin resistance marker but screening for tetracycline- or erythromycin-susceptible colonies. The selected kanamycin-resistant transductants lost their tetracycline or erythromycin resistance, indicating that double-crossover events led to the restoration of the wild-type *trfAB* genes. We observed a ca. 96% cotransduction frequency between kanamycin resistance and the loss of tetracycline resistance or erythromycin resistance with the restoration of the wild-type *trfAB* genes in the Δ*trfA*, Δ*trfB*, and Δ*trfAB* strains, which was expected by the close physical linkage. The restoration of wild-type *trfA* and *trfB* was confirmed by PCR and sequencing. Independent kanamycin-resistant and tetracycline- or erythromycin-susceptible colonies were subsequently tested for teicoplanin resistance.

RESULTS

Differences in genomic contents between clinical Tei^r and Tei^s strains. Comparison of Tei^r strain 14-4 (teicoplanin MIC = 16 to 32 μg/ml) with its Tei^s MRSA parent strain, strain MRGR3 (teicoplanin MIC = 1 to 2 μg/ml), by CGH analysis revealed no detectable difference in intensity in any of the 5,337 oligonucleotide probes used. In contrast, six probe signals were absent from the Tei^s revertant 14-4rev (teicoplanin MIC = 1 μg/ml) compared to the signals obtained with strains 14-4 and MRGR3. All six signals missing from the Tei^s revertant were clustered in a single 1.8-kb chromosomal region. Two putative ORFs, hereafter designated teicoplanin resistance factors *trfA* and *trfB*, respectively, were identified in this region, and these correspond to N315 ordered sequence tag numbers SA0857 and SA0858, respectively (Fig. 1).

To determine the precise limits of the deleted chromosomal region detected in 14-4rev, we sequenced the entire 3-kb region encompassing *trfA* and *trfB* in all three strains. The results are depicted in Fig. 2. Two IS*256* elements at identical positions were present in strains MRGR3 and 14-4: one was inserted upstream of *trfA* and the second IS*256* element was inserted at nucleotide position 872 from the *trfB* start codon, possibly generating a C-terminal 38-amino-acid truncation of the *trfB* gene product. Strikingly, sequence analysis showed that one IS*256* element was absent from strain 14-4rev. The results of alignment and breakpoint analysis were consistent

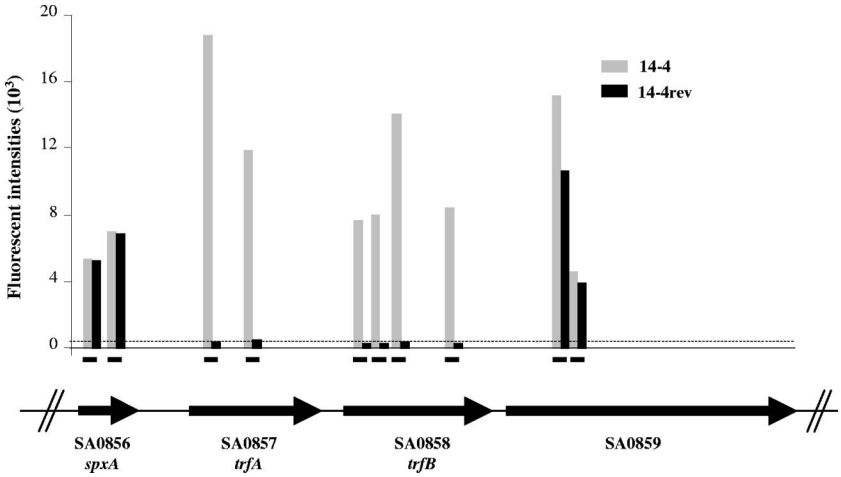

FIG. 1. CGH analysis of Tei^r strain 14-4 and its Tei^s revertant, strain 14-4rev, aligned with the physical map. The probes recognizing *spxA*, *trfA*, *trfB*, and SA0859 are indicated by small black boxes along the horizontal axis. The background intensity is indicated by the dotted line.

with homologous recombination between the surrounding IS*256* elements, resulting in the deletion of both *trfA* and *trfB*.

The *trfA* ORF was predicted to encode a 239-amino-acid protein. Searches for sequences with homology with *trfA* indicated the presence in *Bacillus subtilis* and *Listeria monocytogenes* of genes showing partial similarity and annotated as *mecA* and *ypbH*. The hypothetical *S. aureus* TrfA protein showed 35% identity with MecA1 of *Bacillus subtilis* (SwissProt accession number P37958) and MecA of *Listeria monocytogenes* (SwissProt accession number Q9RGW9). Alignment of the TrfA and MecA1 sequences with the ClustalW program revealed a conserved N-terminal domain (amino acids 1 to 78) with 57% and 52% identities with MecA1 of *B. subtilis* and MecA of *L. monocytogenes*, respectively, and a C-terminal domain (amino acids 106 to 239) with 27% identity with both MecA1 of *B. subtilis* and MecA of *L. monocytogenes*.

The *trfB* ORF was predicted to encode a 328-amino-acid protein. TrfB showed identities of 27% and 24% with YjbF of *B. subtilis* (SwissProt accession number O31604) and Lmo2189 of *L. monocytogenes* (SwissProt accession number Q8Y582), respectively. In *B. subtilis*, YjbF, also called CoiA in *Streptococcus pneumoniae*, is thought to colocalize with proteins involved in competence (22). The functions of *trfA* and *trfB* have not previously been described in *S. aureus*, nor have these genes been detected by proteomic analysis (42).

Since the specific loss of the *trfA* and the *trfB* genes in Tei^s 14-4rev suggested their possible role in the expression of teicoplanin resistance, we examined their roles by use of a genetic approach.

In vitro selection of stable teicoplanin-resistant mutants of *S. aureus* 8325. Despite repeated efforts in our laboratory, MRGR3 and its derivatives have proven refractory to most genetic manipulations. To circumvent this difficulty, we first generated spontaneous teicoplanin-resistant mutants in a genetically tractable background. Subsequently, we used targeted disruption methods to evaluate the contribution of *trfA* or *trfB*, or both, to teicoplanin resistance.

A two-step procedure was used for the selection of stable Tei^r mutants of Tei^s NCTC8325 laboratory strain ISP794 (teicoplanin MIC by macrodilution = 1 μg/ml). Three second-step mutants that were selected on BHIA supplemented with 4 μg/ml of teicoplanin showed reproducible eightfold increases

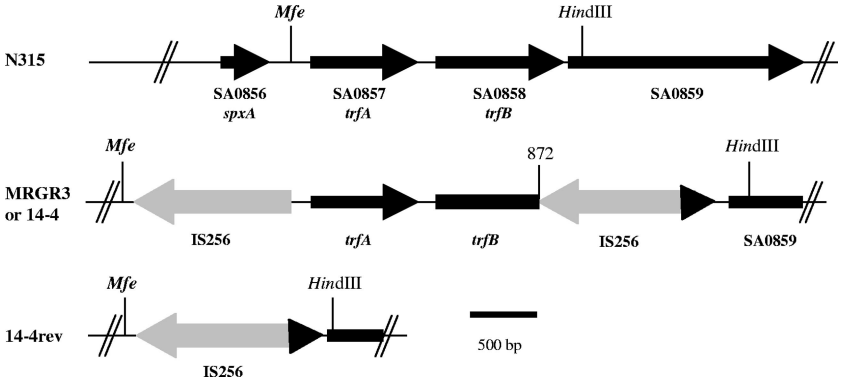

FIG. 2. Schematic representation of the genomic *trfA* and *trfB* region of *S. aureus* strains N315, MRGR3, and 14-4; this region is deleted in strain 14-4rev. This region is localized between the Mfe and HindIII restriction sites at nucleotide positions 518 and 2725, respectively, from the ATG start codon of the upstream *spxA* gene. IS*256* elements are indicated by gray arrows. The second IS*256* element was inserted at nucleotide position 872 from the ATG start codon of the *trfB* gene. Ordered sequence tags are shown by using the annotation from the N315 sequence (25).

in their teicoplanin MICs compared to the MIC for their ISP794 Tei^s parent strain. One of them, strain ISP4-2-1, was chosen for use in further genetic studies.

Phenotypic, antibiogram, and molecular typing analyses confirmed the strong similarity of Tei^r strain ISP4-2-1 with its Tei^s parent, strain ISP794 (data not shown). The growth rate of Tei^r ISP4-2-1 was marginally decreased in MHB compared to that of its Tei^s parent, ISP794 (data not shown). An eightfold increase in the teicoplanin MIC was observed, while a twofold marginal increase in the vancomycin MIC was observed. Multiple passages in antibiotic-free medium did not alter the teicoplanin resistance level measured, as indicated by an MIC assay, suggesting a stable teicoplanin-resistant phenotype.

To analyze Tei^r strain ISP4-2-1 for possible mutations in the promoter or/and the coding regions of the *trfAB* segment which could have arisen during the in vitro selection of Tei^r strain ISP4-2-1, we sequenced the entire chromosomal region encompassing SA0856 to SA0859 of Tei^r ISP4-2-1 and its Tei^s parent, ISP794. No nucleotide sequence changes were detected between Tei^r ISP4-2-1 and Tei^s ISP794.

Effect of *trfAB* mutation on glycopeptide resistance. To evaluate the contribution of *trfA* and *trfB* to teicoplanin resistance, we constructed a *trfAB*-disrupted mutant of Tei^r strain ISP4-2-1 named AR532 (Table 1). As observed by macrodilution MIC analysis and as confirmed by PAP assays, Δ*trfAB* was found to be as susceptible to teicoplanin as its Tei^s parent, ISP794. Indeed, the teicoplanin MICs decreased from 8 μg/ml for ISP4-2-1 to 1 μg/ml for the Δ*trfAB* strain. Furthermore, PAP assays of the Δ*trfAB* strain revealed no residual CFU on MHA supplemented with 2 μg/ml of teicoplanin, whether the strain was tested by spot tests or standard plating assays (Fig. 3A and B). Identical results were obtained when the growth of another of our independently isolated Tei^r ISP794 second-step mutants (ISP4-2-4) and that of its isogenic Δ*trfAB* mutant were compared (data not shown).

To confirm that the loss of teicoplanin resistance was linked with inactivation of *trfA* and *trfB*, restoration experiments were performed (Fig. 4A and B). To facilitate restoration of the wild-type *trfAB* chromosomal sequence by homologous recombination, we first engineered the donor derivative strain of Tei^r ISP4-2-1, named AR548, by inserting a kanamycin resistance determinant ca. 2 kb downstream of the *trfA* and the *trfB* genes. Control experiments ruled out any detectable influence of the kanamycin resistance cassette carried by AR548 on the teicoplanin resistance phenotype compared to that on its parent strain, Tei^r ISP4-2-1 (Fig. 4B). We expected that screening for tetracycline-susceptible, kanamycin-resistant phage transductants from donor strain AR548 to Δ*trfAB* would restore wild-type *trfAB* genes, and this was confirmed by PCR. Macrodilution MIC and PAP assays showed the complete restoration of teicoplanin resistance in the resulting strain in which *trfA* and *trfB* were restored (strain AR561), and the teicoplanin resistance was equivalent to that observed in Tei^r ISP4-2-1 (Fig. 3A).

The teicoplanin susceptibility of the Δ*trfAB* strain was further evaluated by determination of the relative ECF on agar plates supplemented with different concentrations of teicoplanin. When the residual CFU on teicoplanin-containing agar was normalized by the CFU on antibiotic-free MHA, the viable counts of the Δ*trfAB* strain showed a >5-$\log_{10}$-unit reduction on teicoplanin-containing agar compared to the viable counts of Tei^r ISP4-2-1. In contrast, no significant difference in the ECF of Tei^r ISP4-2-1 and the strain in which *trfA* and *trfB* were restored was observed (Table 3). Collectively, teicoplanin sus-

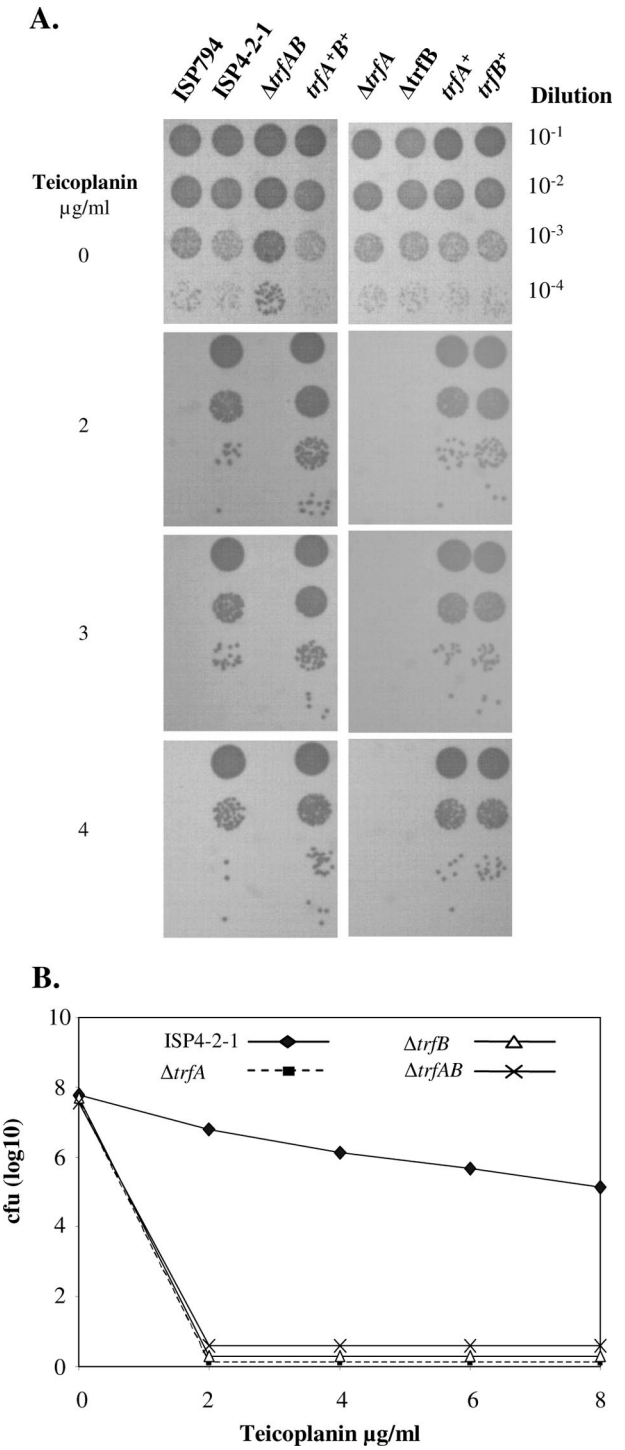

FIG. 3. Effects of the *trfA* and/or the *trfB* gene mutation on teicoplanin resistance. (A) Spot plating population analysis of strain ISP794 and its derivatives. Spot dilutions are indicated on the right. The first 10-μl spot dilution corresponds to 1×10^5 CFU. (B) PAPs obtained by standard plating on teicoplanin-containing MHB.

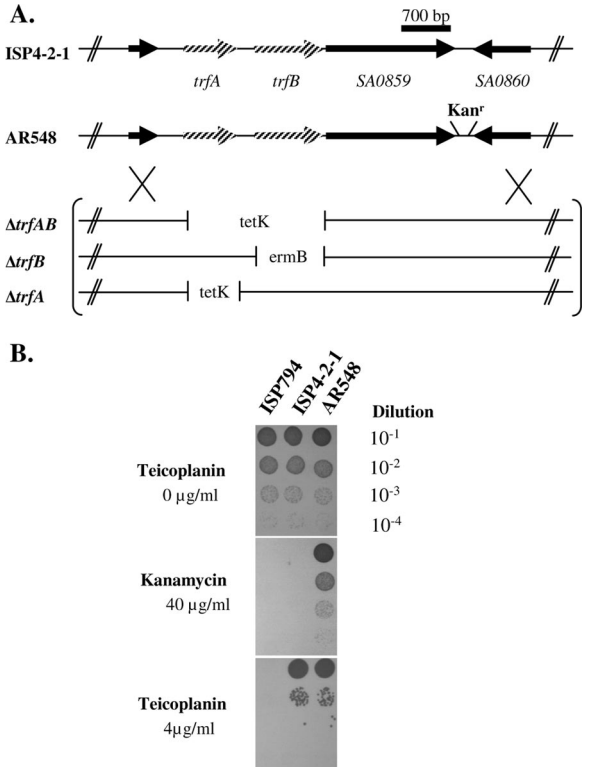

FIG. 4. (A) Schematic representation of the procedure used to restore the Δ*trfA* and/or the Δ*trfB* gene. Strain AR548 was generated by inserting a kanamycin resistance determinant in strain ISP4-2-1 between the SA0859 and the SA0860 genes. The different mutants, Δ*trfAB*, Δ*trfB*, and Δ*trfA*, were restored by the transduction of wild-type chromosomal DNA from the AR548 strain. Double crossovers were obtained by screening for tetracycline- or erythromycin-susceptible but kanamycin-resistant clones. (B) Insertion of the kanamycin resistance marker near the *trfAB* genes in strain ISP4-2-1 does not affect the teicoplanin-resistant phenotype. Spot plating population analysis of ISP794, ISP4-2-1, and its kanamycin-resistant derivative (strain AR548) on kanamycin and teicoplanin agar. The first 10-μl spot dilution corresponds to 1×10^5 CFU.

ceptibility testing on agar medium further supported the key role of the *trfAB* genes on teicoplanin resistance.

The impact of Δ*trfAB* on the twofold marginal increase in the vancomycin MIC of strain ISP4-2-1 was also examined. The relative ECF of the Δ*trfAB* strain showed a 3-$\log_{10}$-unit reduction on agar supplemented with 2 μg/ml of vancomycin compared to the ECF of Tei^r ISP4-2-1. In contrast, no significant reduction in the ECF of Tei^r ISP4-2-1 compared to that of the strain in which *trfA* and *trfB* were restored was observed (data not shown).

Collectively, we conclude that the deletion of the *trfAB* sequences abolishes not only teicoplanin resistance but also vancomycin resistance, although to a lesser degree, in strain ISP4-2-1.

Individual contributions of *trfA* and *trfB* to teicoplanin resistance. To assess the respective roles of *trfA* and *trfB* in teicoplanin resistance, we next assayed the impacts of separate Δ*trfA* and Δ*trfB* mutations on susceptibility to teicoplanin. Targeted disruptions were engineered in *trfA* or *trfB* in strain ISP4-2-1. Our results showed that each mutant displayed reduced teicoplanin MICs equivalent to that of the Tei^s parent strain ISP794 (1 μg/ml). Furthermore, in PAP assays, neither the Δ*trfA* mutant (AR612) nor the Δ*trfB* mutant (AR618) yielded a single CFU on 2 μg/ml of teicoplanin-containing agar; the numbers of CFU were determined by spot tests or the standard plating assay (Fig. 3A and B). Analysis of the teicoplanin resistance of strains in which *trfA* was restored (strain AR627) and *trfB* was restored (strain AR628) showed PAPs and MICs (8 μg/ml) equivalent to those of Tei^r ISP4-2-1 (Fig. 3A).

ECF analysis of the Δ*trfA* and the Δ*trfB* mutants on agar plates supplemented with different concentrations of teicoplanin showed a >5-$\log_{10}$-unit reduction in ECF compared to that of Tei^r ISP4-2-1. No difference in CFU was observed when the numbers of CFU of Tei^r ISP4-2-1 and the CFU of either the strain in which *trfA* was restored or the strain in which *trfB* was restored were compared (Table 3).

To observe the impact of *trfA* and *trfB* deletions on the phenotypic detection of teicoplanin susceptibility or resistance in an optimal way, we consistently tested freshly prepared mutants obtained by bacteriophage backcrossing. Indeed, we initially noticed that prolonged subculture of the Δ*trfA* strain resulted in the emergence of colonies growing on agar supplemented with low levels (2 μg/ml) of teicoplanin, which suggested the possible emergence of unlinked compensatory mutations. PCR analysis of the isolates from two independent colonies confirmed the presence of the Δ*trfA* deletion, suggesting that the bacteria rapidly adapted to the loss of *trfA*. In contrast, we never observed the emergence of compensatory mutations in either Δ*trfB* or Δ*trfAB* mutants under our laboratory conditions. We conclude from the results of that analysis that both *trfA* and *trfB* contribute to the maintenance of teicoplanin resistance in strain Tei^r ISP4-2-1. Nevertheless, the more stable Tei^s phenotype of the Δ*trfB* and Δ*trfAB* strains indicates that they are more suitable for use in future genetic studies.

Impact of Δ*trfAB* in NARSA GISA reference strain NRS3. NRS3 is a clinical MRSA reference strain that exhibits a pro-

TABLE 3. ECF on different concentrations of teicoplanin-supplemented agar

Strain	Relevant genotype	ECF[a] with teicoplanin at:		
		2 μg/ml	4 μg/ml	8 μg/ml
ISP794[b]		$<10^{-7}$	$<10^{-7}$	$<10^{-7}$
ISP4-2-1		2×10^{-2}	1×10^{-2}	2×10^{-3}
AR532[b]	Δ*trfAB*::*tetK*	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$
AR561	*trfA* and *trfB* positive	5×10^{-2}	1×10^{-2}	1×10^{-4}
AR612[b]	Δ*trfA*::*tetK*	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$
AR627	*trfA* positive	4×10^{-2}	2×10^{-2}	ND[c]
AR618[b]	Δ*trfB*::*ermB*	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$
AR628	*trfB* positive	9×10^{-2}	1×10^{-2}	ND
AR619	Δ*trfAB*::*tetK*	2×10^{-1}	7×10^{-4}	$<10^{-7}$
NRS3		6×10^{-1}	4×10^{-1}	4×10^{-2}

[a] The number of survivors in the presence of teicoplanin at each concentration was normalized to the number of bacteria plated on agar without teicoplanin. Data are reported for four independent experiments.

[b] Strains showing no single CFU on agar supplemented with teicoplanin at concentrations ranging from 2 to 8 μg/ml. The viable counts on agar without teicoplanin were $>10^7$ CFU/ml.

[c] ND, not determined.

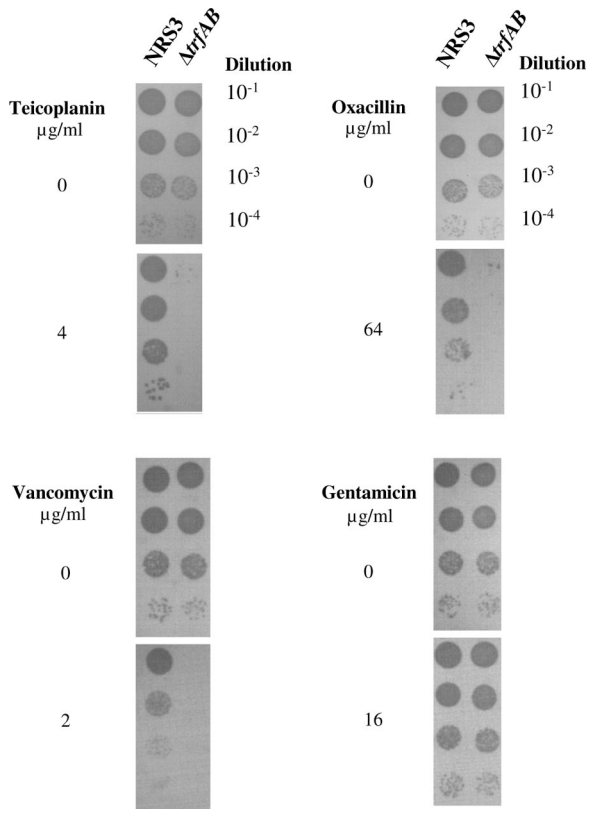

FIG. 5. Effect of Δ*trfAB* gene mutation on various antibiotic resistance determinants in strain NRS3. The results of spot plating population analysis of NRS3 and NRS3Δ*trfAB* on teicoplanin-, vancomycin-, oxacillin-, and gentamicin-supplemented agar are shown. The serial spot dilutions are indicated on the right margin. The first 10-μl spot dilution corresponds to 1×10^5 CFU.

nounced GISA phenotype (vancomycin and teicoplanin MICs = 8 μg/ml; oxacillin MIC > 32 μg/ml; gentamicin MIC > 16 μg/ml). The impact of Δ*trfAB* on teicoplanin resistance was therefore evaluated by transducing the Δ*trfAB* mutation into strain NRS3. A twofold reduction in the teicoplanin macrodilution MIC for the NRS3 Δ*trfAB* mutant (strain AR619) compared to that for its isogenic parent NRS3 was recorded. A more striking effect was observed in PAP assays on agar supplemented with 4 μg/ml of teicoplanin (Fig. 5), in which the relative ECF showed a 3-$\log_{10}$-unit reduction in CFU compared to the numbers of CFU for NRS3. A higher reduction was observed on agar supplemented with 8 μg/ml of teicoplanin, in which the relative ECF showed a >5-$\log_{10}$-unit reduction in CFU (Table 3).

Since NRS3 displays multiple resistance to several other antibiotics, e.g., vancomycin, oxacillin, and gentamicin, NRS3 Δ*trfAB* was also tested for changes in its susceptibility to each of these antibiotics. PAP assays revealed an increased susceptibility of NRS3 Δ*trfAB* compared to that of NRS3 on agar containing 2 μg/ml of vancomycin, in which the relative ECF showed a >5-$\log_{10}$-unit reduction in CFU. NRS3 Δ*trfAB* was also more susceptible to oxacillin, in which the relative ECF showed a 3-$\log_{10}$-unit reduction in CFU with oxacillin at 64 μg/ml. In contrast, no difference in the levels of expression of gentamicin resistance was observed between NRS3 Δ*trfAB* and its parent strain, strain NRS3, when different gentamicin concentrations were tested (Fig. 5).

Taken together, these results provide evidence that *trfAB* not only contributes to glycopeptide resistance but also modulates oxacillin resistance.

Role of *trfAB* in teicoplanin resistance emergence. To analyze the role of the *trfAB* region in the emergence of teicoplanin resistance, as opposed to its effects on preexisting resistance, we constructed a Δ*trfAB* mutant of the Tei^s ISP794 strain (strain AR596) and analyzed if the frequency of selection of teicoplanin-resistant mutants was detectably affected in the absence of *trfAB*. We observed that both the Tei^s ISP794 parent strain and ISP794 Δ*trfAB* were able to form colonies after 48 h of growth when ca. 3×10^8 bacteria were plated on agar plates containing 2 μg/ml teicoplanin. However, we observed a consistent reduction in the frequency of emergence of teicoplanin mutants selected from ISP794 Δ*trfAB* of >80% compared to the frequency of emergence of teicoplanin mutants selected from parent strain ISP794 in six independent trials. Since the selection of teicoplanin-resistant mutants was markedly affected by the absence of *trfAB*, but Tei^r mutants could still be selected, we conclude that *trfAB* is needed for some, but not all, pathways that lead to the emergence of low-level glycopeptide resistance.

DISCUSSION

In this study, the use of combined genomic and genetic approaches with *S. aureus* identified two genes, *trfA* and *trfB*, which are involved in resistance to two classes of cell-wall-active antibiotics. Engineered insertional disruption of either or both *trfA* and *trfB* led to significant reductions in the levels of both teicoplanin and vancomycin resistance in laboratory strain ISP794, which was previously selected for stable low-level glycopeptide resistance. The disruption of *trfAB* also significantly affected, but did not entirely block, the emergence of low-level teicoplanin resistant mutants from parental Tei^s strain ISP794. We suspect that other alternative pathways might lead to the selection of low-level glycopeptide resistance. The disruption of *trfAB* also showed that the locus was important for both glycopeptide and oxacillin resistance in a NARSA reference MRSA strain and vancomycin-intermediate *S. aureus* strain NRS3. Since the means of regulation of *trfA* and *trfB* expression is unknown, we preferred to restore *trfA*, *trfB*, or *trfAB* mutants by allelic exchange with their wild-type copies of *trfAB* rather than by multicopy plasmid complementation, which might have altered gene expression. The results of preliminary studies indicated that the *trfA* mutation exerts no polar effect on *trfB* transcription when it was examined by quantitative reverse transcription-PCR and Northern blotting. Furthermore, *trfA* and *trfB* do not comprise an operon, as determined by Northern blotting (data not shown).

Besides the mutation in *trfA* or *trfB* described in this study, mutations in several other genes, for example, *vraRS*, *graRS*, and *ccpA* (24, 31, 35, 43), are known to confer the loss of glycopeptide resistance. In contrast, increased teicoplanin resistance is associated with the inactivation of *tcaA* (29).

The precise genetic change(s) underlying the emergence of teicoplanin resistance in strain ISP4-2-1 in our study is presently unknown. Mutations in several genetic loci (*sigB*, *agr*,

vraSR, *graSR*, *prsA*, *yycF*) have been shown to contribute to the emergence of glycopeptide resistance during in vitro studies as well as during the acquisition of vancomycin resistance in clinical isolates of patients treated with glycopeptides (2, 18, 33).

The functions of *trfA* and *trfB* have not been previously described in *S. aureus*. The conceptual translation product of *trfA* is most closely related to that of the *mecA* genes of *B. subtilis* and *Listeria monocytogenes*, which have no relation to the *mecA* gene found in the staphylococcal chromosomal cassette *mec* element in *S. aureus*. In *B. subtilis*, MecA plays a significant regulatory role in competence development, motility, and autolysis (13, 39), while in *L. monocytogenes*, MecA functions to downregulate SpvA, a surface virulence-associated protein (3). Proteins whose amino acid sequences have high degrees of similarity to the amino acid sequence of the conceptual translation product of *S. aureus trfB* have been described in both *B. subtilis*, in which it is called YjbF, and its ortholog, CoiA (competence-inducible A), in *Streptococcus pneumoniae* (38). Studies with both organisms suggest a role for CoiA/YjbF in competence for genetic transformation (12). (22).

In *B. subtilis*, MecA functions as an adaptor for regulated proteolysis of the transcription factor ComK through interaction with the AAA+ Hsp100/Clp ATPase family member ClpC. Besides regulating genes involved in the competence state, *B. subtilis* ComK also affects cell wall hydrolases and survival under conditions of stress (1, 27). MecA possesses a second important function by ensuring the proper oligomeric assembly of ClpC (20). ClpC is known to regulate the proteolytic turnover of ComK, Spx, CtsR, and MurAA, which play key roles in competence, redox sensing, heat shock regulation, and the first committed step of cell wall biosynthesis, respectively (21, 23, 34, 47).

In *S. aureus*, no functional role has been established for TrfA and SA0882, which are considered MecA and ComK orthologs, respectively. Nevertheless, the role of ClpC was shown to be important in *S. aureus* for biofilm formation, stress tolerance, and regulation of the tricarboxylic acid cycle (6, 14). As far as we know, the precise role of ClpC in response to drug exposure and antibiotic resistance is not well defined. It is tempting to speculate that our *trfA* mutation alters ClpC assembly and therefore affects downstream proteolytic regulatory processes in *S. aureus*. Furthermore, the widely documented evidence implicating *trfA* and *trfB* homologs in competence regulation in other organisms suggests a possible link between the *S. aureus* putative competence machinery and low-level glycopeptide resistance.

In conclusion, our study has documented the important roles of two novel loci in endogenous, low-level glycopeptide resistance in *S. aureus*. Further studies are warranted to elucidate their precise molecular function(s). The putative roles of TrfA and TrfB and their homology with factors that play key roles in competence development and chaperone functions offer exciting perspectives. A worthwhile area of antibiotic research would appear to be the posttranscriptional processing by the molecular chaperones and the regulated proteolysis of proteins destined to remodel the cell wall, transit the plasma membrane, and clear drug-related damage.

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